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TITLE: Genomic and Expression Profiling of Benign & Malignant Nerve Sheath Tumors in Neurofibromatosis Patients

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14. ABSTRACT

The goal of the study is to identify genes that will serve as molecular markers for progression of neurofibroma to MPNST, and to identify potential therapeutic targets. miRNA expression profiling was performed on 6 cases of MPNSTs, and 7 cases of synovial sarcomas. By using unsupervised hierarchical clustering most tumors were grouped together according to tumor type. Subsequent analyses using Significance Analysis of Microarrays (SAM) identified miRNAs that differentiate between MPNSTs and synovial sarcoma (SS). To develop a cell line model for MPNSTs, global gene expression profiles for cell lines established from 3 primary MPNST and SS tumor tissues was carried out and their expression profiles were compared with other sarcomas. A large tissue microarray (TMA) containing about 200 nerve sheath tumors was used to test for EGFR expression by IHC. Neoplasms in which the majority of samples showed high expression by IHC included MPNST (83% of NF1-associated and 77% of sporadic), 73% of plexiform neurofibroma, 100% diffuse neurofibroma and 93% of SS.

15. SUBJECT TERMS
miRNA microarrays, expression profiling, tissue microarrays, Immunohistochemistry

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Title: Genomic and Expression Pr ofiling of Benign and Malignant Nerve Sheath Tumors in Neurofibromatosis Patients.

INTRODUCTION

Neurofibromatosis Type 1 (NF1) affects 1:3000 live births and can be inherited as an autosomal dominant trait that is caused by mutations in the NF1 gene or acquired by a de novo mutation in this gene. There is a significant risk of developing malignancies, the vast majority of which are malignant peripheral nerve sheath tumors (MPNST). The incidence of malignant transformation in patients with NF1 is ~5%. These tumors are aggressive sarcomas and are associated with a 5-year survival rate of ~40%. Malignant transformation is not easy to identify but can be associated with the occurrence of pain and growth in a preexisting neurofibroma. The number of neurofibromas that patients typically have and their location make screening for transformation to MPNST difficult and therefore the MPNSTs are often found at a late clinical stage (Weiss and Goldbum, 2001).

Our extensive messenger RNA expression profile data show unexplained down regulation of gene expression for many genes as being one of the most prominent features of the transition of NF to MPNST (see 3rd annual report). We hypothesize that microRNA (miRNA) expression differences, as yet unstudied in NF and MPNST, may play a central role in these findings. Recent studies suggest that knowledge of miRNA expression patterns in cancer may have substantial value for diagnostic and prognostic determinations as well as for eventual therapeutic intervention (Calin and Croce, 2006; Calin *et al.*, 2005; Esquela-Kerscher and Slack, 2006). MicroRNAs (miRNAs) are a class of conserved, noncoding RNAs, composed of single-stranded RNAs of ~ 19-22 nucleotides. These regulatory small RNAs play a role in gene regulation (Lau *et al.*, 2001) through RNA interference (RNAi) mechanisms. Functionally, miRNAs can inhibit the protein synthesis from a wide variety of mRNAs through either inhibition of translation by imperfect base-pairing with their target mRNA at the 3'UTR or by inducing cleavage of target mRNA through perfect complementary sequence base-pairing (Bartel, 2004; Doench and Sharp, 2004).

In the last year we have extensively worked on standardization of microRNA expression profiling using the microRNA microarrays printed at the Stanford functional genome facility. We have profiled the miRNA expression for 6 MPNSTs and 7 synovial sarcomas (a tumor in the differential diagnosis). In collaboration with the laboratory of Dr. Andy Fire, who recently was awarded the Nobel Prize for his work on miRNA, we also cloned and sequenced the small

RNA from a MPNST and a neurofibroma case; we also cloned miRNAs from 2 cases of synovial sarcomas.

BODY

Specific aim 1: Genome wide search for genes in nerve sheath tumor

A1. Gene expression profiling of MPNST compared to Neurofibromas and other soft tissue tumors.

We have determined the gene expression signature for benign and malignant peripheral nerve sheath tumors and found that the major trend in transformation from neurofibroma to MPNST consists of loss of expression of a large number of genes, rather than widespread increase in gene expression. Our gene expression analysis indicates that malignant transformation in PNSTs involves multiple molecular events reflected in expression signatures for cell proliferation and p53 inactivation. Deregulation of genes associated with cell adhesion function and elevated expression of genes implicated in tumor metastasis is seen in the majority of MPNSTs. In addition, MPNSTs are characterized by loss of expression of genes associateed with signaling pathways. Evaluation of EGFR protein expression using a tissue microarray was used to validate the gene array data.

The details of this study are in the manuscript "Genome-wide transcriptome analysis identifies gene signature for malignant transformation in peripheral nerve sheath tumors " which is submitted for publication and is attached as an appendix (Appendix 1) to this final report.

B1. Standardization of miRNA expression profiling

miRNA microarrays used in the study were printed at the Stanford Functional Genomics Facility. The arrays contained a total of 668 probes spotted in duplicate. The 668 probes represent 328 known human miRNAs, 113 mouse miRNAs, 45 rat miRNAs, 154 predicted human miRNAs and 28 control probes (Ambion, Austin, Texas). Since the regular (TRIzol) method eliminates the small RNA species, the miRNA isolation procedure from frozen tissue samples had to be modified. Our objective was to isolate the total RNAs that included the small RNA species so that the RNA obtained by this procedure will be used in both messenger RNA arrays and for miRNA arrays. In our final protocol, total RNA is extracted from frozen tumor samples using the mirVana^{TM} miRNA isolation kit (Ambion, Austin, Texas) with modifications to isolate up to 250 ug of total RNA. Further, using a proper 'Reference RNA' for the array expression is an important step in developing a protocol for miRNA arrays. After testing reference RNA samples form many vendors, we identified reference RNA (XpressRef^{TM} Universal Total RNA) obtained from SuperArray (Frederick, MD) as having a high quality and quantity of small RNA species. miRNA was further enriched from 25 μg of total RNA using a microcon YM-100 column (Millipore, Billerica, MA), and indirectly labeled with Cy3 or Cy5 amine reactive dyes (Amersham, Buckinghamshire, UK) using mirVana^{TM} miRNA labeling kit (Ambion). Post-processing of arrays and washing conditions were modified to obtain high quality of data. Hybridization was at 42^{0}C for 12-16 hrs. Arrays were washed and

immediately scanned using a GenePix 4000B array scanner (Axon Instruments, Foster City, CA).

B2. miRNA expression profiling and analysis

The miRNA expression profile for 6 MPNST cases was determined and compared to 7 synovial sarcomas (SS). Fluorescence ratios (sample/reference) were calculated using GenePix software and miRNA arrays were normalized. To limit the measurement errors, only miRNA spots with a ratio of signal over background of at least 2 in both Cy3 and Cy5 channels were included. Further, miRNA spots were filtered based on those where expression levels differed by at least four-fold in at least two arrays. Finally miRNA spots with 80% good data were selected. As a first step in the analysis, we asked whether the miRNA expression signatures of these tumor types were distinct. The 60 miRNAs that met the filtering criteria were subjected to hierarchical clustering among these 13 sarcomas in an unsupervised manner. The clustering algorithm grouped both miRNAs and samples into clusters based on overall similarity in miRNA expression pattern without prior knowledge of sample identity. Clustering based on the 60 miRNAs revealed substantive distinctions in overrepresented and underrepresented miRNAs among the tumors (Figure 1). As is evident from the dendrogram at the top of the heat map, the tumors clustered into 2 main groups, whereby all MPNSTs and SS separated into two distinct groups.

B3. Significance Analysis of Microarrays

Using SAM analysis, we identified the miRNAs that distinguished MPNST from synovial sarcomas. A total of 14 miRNAs showed significant relative overrepresentation in MPNSTs compared to SS. Five miRNAs showed relative underrepresentation in MPNSTs. The results for the top miRNAs of each class with <2% FDR (false discovery rate) are detailed in Table 1.

B4. Small RNA isolation, cloning and analysis

Small RNA extracted using mirVana^{TM} miRNA isolation kit (Ambion) was used as starting material for cloning procedure of Lau *et al (Lau et al., 2001)* with slight modifications. Purified small RNAs were ligated with pre-adenylylated 3'-adaptor oligonucleotide, gel purified, and subjected to a second ligase reaction with a 5'-adaptor oligonucleotide. The gel-purified, doubly-ligated RNA was reversed transcribed using Superscript II (Invitrogen, Carlsbad, CA) and RT primer, followed by PCR amplification using the RT primer and a forward primer. A second PCR was performed using the RT primer and a second forward primer. The PCR product was purified by phenol/chloroform extractions and then digested with Ban I (NEB, Beverly, MA) for concatemerization using T4 DNA ligase (NEB). Concatamers ranging from 600-1000 bp were isolated from a low-melting-point agarose gel, processed with Taq polymerase, and cloned into the pCR4-TOPO vector using the TOPO TA cloning kit (Invitrogen). Colony PCR was performed

using the M13 forward and reverse primers, and the PCR products were prepared for sequencing using shrimp alkaline phosphatase and exonuclease I (USB Corporation, Cleveland, Ohio). Small RNAs obtained by cloning were compared with functionally annotated sequences using BLAST (blastn, http://www.ncbi.nlm.nih.gov/blast/), BLAT (http://genome.ucsc.edu), miRBase and simple text searches. For each cloned small RNA, the best alignments to a functionally annotated sequence (not more than one error) were used to assign a functional category to the small RNA. Putative novel small RNAs were analyzed using mfold version 3.2 (Zuker, 2003) to identify potential precursor structures.

miRNA were cloned and sequenced from small RNA libraries of 4 sarcomas (1 MPNST, 1 NF and 2 SS) tissues. A total of 1681 small RNA clones were sequenced, of which 94 could be annotated as known miRNAs (Tables 2 and 3).

C1. Gene expression profiles of MPNST and synovial sarcoma cell lines established from primary tumors.

Cell lines derived from fresh tumors have been widely used as experimental models to understand the biology of tumor progression and other treatment strategies. Dr Jonathan Fletcher at Brigham and Women's Hospital, Harvard Medical School has established 31 sarcoma cell lines that included 3 MPNSTs and 3 synovial sarcomas. In collaboration with his group we have characterized the global gene expression profiles for 3 MPNST and 3 synovial sarcoma cell lines using HEEBO arrays and compared the expression profiles of these tumors with the other sarcoma cell lines. Unsupervised hierarchical analysis clustered the gene array data from 31 cell lines broadly clustered the cell lines based on the tumor-type (Figure 2). Two of the three MPNST cell lines clustered together and all the 3 synovial sarcoma cell line formed a tight cluster. The preliminary data on the gene expression profiles of these cell lines was presented as a poster in the 2007 annual meeting of United States and Canadian Academy of Pathology (USCAP; see appendix 2). We are currently in the process of comparing the data generated from primary MPNST and SS tumors to the MPNST and SS cell lines.

Specific Aim 2: Validation of candidate genes on large numbers of cases using immunohistochemistry and *in situ* hybridization on TMA.

TMAs form excellent tools to validate and extend findings from gene array studies since paraffin-embedded archival material is much easier to collect than fresh frozen material. The TMA was constructed as part of a close collaboration between the Stanford group and the funded collaborators at University of Washington (Brian Rubin) and University of British Columbia (Torsten Nielsen) (see annual reports 2 and 3). As a result we now have access to what to our knowledge is the largest TMA of nerve sheath tumors. This TMA (TA-138) contains 68 MPNSTs, 42 neurofibromas, 22 schwannomas and 15 synovial sarcomas. All the cases were represented in duplicate cores of 0.6mm diameter.

Attempts to develop ISH probes for miRNA

We have had good success confirming gene expression profiling studies using non-radioactive miRNA probes for in situ hybridization (ISH) in formalin-fixed paraffin-embedded (FFPE) tissue. While the cloning experiments described above form an independent validation of the array-based miRNA studies further confirmation is obtainable if we could use ISH for miRNA or FFPE. This would also have the benefit of allowing us to precisely identify the cell type for which the miRNA signal from which this miRNA signal originates that we see in lysates of whole tumors. In the past 2 years we have spent considerable effort in optimizing our ISH protocol for mRNA to one that could detect mirRNA.

Since miRNA species are extremely short (19-22 nucleotides) we decided to generate LNA probes for these molecules. LNA has a much higher melting temperature than RNA and thus seems optimal for these studies.

We have been able to obtain very strong signals using these probes but background problems continued to frustrate us in this approach. Most of our experience is based on work with LNA probes miR143 and miR21. Despite extensive efforts to get this technique working we have been unable to generate results that we trust to be without artifacts. Ongoing review f the literature and discussions with other investigators has shown that this approach may have inherent difficulties that may be impossible to overcome. In recent months we have decided to abandon this approach and to focus instead on the combination of laser capture microdissection paired with quantitative PCR to resolve the localization of the miRNA expression. If successful, this technique then can be used as a positive control for the ISH approach.

EGFR expression studies using nerve sheath tumor tissue array.

Epidermal growth factor receptor (EGFR), located at 7p12, is a member of a protein tyrosine kinase family and functions as a cell cycle regulatory protein. The receptor is activated by the binding of epidermal growth factor (EGF) and transforming growth factor, which leads to receptor autophosphorylation resulting in cell proliferaton and cell survival through inhibition of apoptosis and promotion of angiogenesis. The multiple cellular signaling interactions of EGFR biology allows for potential EGFR inhibitors to counteract cellular proliferation, angiogenesis, invasion and metastasis. From the gene array data we have identified EGFR that was highly expressed in a significant subset of MPNSTs. Using TA-138 we have studied the differential expression of EGFR in a larger number of cases of PNSTs (see the attached manuscript). Figure 3A-D shows examples of EGFR staining in PNSTs showing 3+ reactivity to EGFR, with >50% of the tumor cells having intense membrane staining. Figure 4 shows an example of EGFR amplification in MPNST. The detailed analyses of EGFR expression in PNSTs are given in Appendix 3.

KEY RESEARCH ACCOMPLISHMENTS

1. Genome wide expression profiling of MPNST and Neurofibromas compared to large set of soft tissue tumors (Appendix1).

2. Standardization of miRNA expression profiling using miRNA microarrays

- Bioinformatical and statistical analyses.
- Identification of miRNAs that distinguish MPNSTs from synovial sarcomas

3. EGFR expression profiling in PNSTs.

4. Global gene expression profiling of novel MPNST and SS cell lines established from primary tumors

REPORTABLE OUTCOMES:

Publication

AJ Larson, E Downs-Kelly, M Skacel, RR Tubbs, BP Rubin, M van de Rijn, RB West, C Corless, A Chiesa and JR Goldblum. Epidermal growth factor receptor (EGFR) expression and gene amplification spectrum of spindle cell soft tissue neoplasms. A fluorescence in situ hybridization (FISH) and immunohistochemical study (in press 2008).

Abstract
Poster presentation at the 2007annual meeting of USCAP.

CONCLUSIONS

In the past years we performed gene expression profiling using Stanford cDNA and HEEBO arrays on a large number of MPNST, neurofibromas and other soft tissue tumors. These studies showed a remarkable downregulated on a large number of genes in MPNST compared to Neurofibromas (Appendix 1). IN a first attempt to determine the reason for this downregulation we decided to study the miRNA expression profile of nerve sheath tumors. We performed miRNA microarray analysis on 6 MPNSTs and 7 synovial sarcomas. We believe that the study of miRNA expression in these tumors may lead to a better understanding of the differences in gene expression profiles we observed using our mRNA expression profiling studies.

We have carried out extensive bioinformatic analysis to annotate the miRNAs and we are preparing to study a larger number of tumors.

We believe that in addition to explaining the biology of NF-MPNST transformation miRNAs can be exploited to be used in the diagnosis of MPNSTs

Extensive attempts were made to optimize methods to use LNA based ISH on FFPE tissues. These studies have not led to success

TMAs were used to study the expression of EGFR in PNSTs and showed that the disparity between EGFR protein overexpression and the paucity of EGFR gene amplification may be secondary to some post-transcriptional modification. The finding of EGFR overexpression, especially in malignant neoplasms,

deserves further study as EGFR antagonists may be of benefit to patients with soft tissue tumors that express and are dependent on EGFR.

Addendum final report DOD grant: DAMD17-03-1-0297

Below I have addressed the comments of the reviewer of our initial final report dated 7-6-08.

The reviewer request a step by step discussion of the original statement of work:

STATEMENT OF WORK

CONTRACTUAL ISSUES: Information provided in this final report supports the following:

Task 1	Months 1-4	Completed
Task 2	Months 5-24	Completed
Task 3	Months 10-28	Completed
Task 4	Months 12-48	Completed
Task 5	Months 5-12	Completed
Task 6	Months 12-48	Not completed
Task 7	Months 24-48	Not completed
Task 8	Months 36-48	Not completed

Task 1 Collect frozen tissue samples and paraffin-embedded specimens from our collaborators and coinvestigators. Centralize samples at Stanford University. Confirmation of frozen sample identity by frozen tissue section histology. Confirmation of diagnoses and clinical histories, through conference phone calls and image sharing through the Internet. Months 1-4.
COMPLETED
A large number of cases were collected and histologically analyzed.

Task 2 Expression profiling of 20 malignant peripheral nerve sheath tumors, 20 neurofibromas, 10 schwannomas, 20 synovial sarcomas, and smaller numbers of other tumors in the differential diagnosis of MPNST on 40,000 element gene microarrays. Months 5-24.
COMPLETED
See report.

Task 3 Array-based comparative genomic hybridization (aCGH) on the same set of cases that are described under Task 2 using the same type of gene

microarrays, to determine a high resolution map of gene copy number gains and losses in MPNST and neurofibroma. Recent data from our laboratory have shown that DNA isolated from archival paraffin embedded samples can also be used in this method. Thus, we will significantly increase the dataset by performing aCGH not only on fresh frozen material, but also on larger numbers of malignant peripheral nerve sheath tumors, neurofibromas, and schwannomas from paraffin-embedded, formalin-fixed tissue. The data obtained in Task 2 and Task 3 will be immediately available to all collaborators through the Stanford Microarray Database. Months 10-28.
COMPLETED
See report.

Task 4 A1. Ongoing and iterative analysis of gene expression data set combined with comparative genomic hybridization data set to look for markers that are differentially expressed in neurofibromas versus malignant peripheral nerve sheath tumors, and for which the genes are differentially amplified or deleted in the same tumors. Find shared genes in both groups and focus on these genes as tumor progression markers. Months 12-48. A2. Start search for potential secreted markers to monitor progression of neurofibroma through MPNST by analyzing predicted amino acid sequence of the genes identified in Tasks 2,3. Months 24-48. B. Search for differential diagnostic markers between malignant peripheral nerve sheath tumors vs. other soft tissue tumors. Months 12-36.
COMPLETED
Despite and extensive search we could not identify reliable differential diagnostic markers OR secreted proteins in our dataset.

Task 5 Building of tissue microarrays (TMAs) with large numbers of tumors from each category and inclusion of a large number of other soft tissue tumors, incorporating the already existing 400 case tissue microarray at Stanford and an 83 case TMA (including synovial sarcomas, MPNST, and other tumors in their differential diagnosis) made by Torsten Nielsen. The goal is to have over 1,000 soft tissue tumors represented in TMAs with approximately 100-200 peripheral nerve sheath tumors isolated from patient with and without neurofibromatosis. Months 5-12.
COMPLETED
See report.

Task 6 Validate the findings from Task 4A, -B on much larger number of cases using immunohistochemistry on tissue microarrays, using at least 200 candidate markers. Existing commercial antibodies will be used in the first experiments. Custom made rabbit antisera will be generated against gene products for which no commercial antibodies exist. Immunohistochemical staining will be performed as an iterative process van de Rijn, Matt Log #NF020008 6 throughout this study and stained sections will be first analyzed under the microscope and the data thus obtained will be analyzed in a custom-made software program we have developed. Subsequently stained tissue microarray slides will be scanned for

digital images of each tissue core using the BLISS computerized microscope system after which re-analysis and comparison with other existing markers will occur using the same custom-made software. Using this method our coinvestigators and collaborators will have direct access to all stained tissue core sections through the Internet, allowing for continuous collaboration without the need to send the immunohistochemical studies to each individual collaborator. Clinical follow-up will be obtained for MPNST occurring in patients with neurofibromatosis and those that occur in patients without this disorder. Immunohistochemical staining profiles for specific markers will be correlated with clinical outcome to identify prognostic markers. Months 12-48.
NOT COMPLETED
Despite an intensive and global search we could not identify markers that fulfilled the criteria stated in Task 4.

Task 7 In the search for a serological marker, we will focus on genes that based on DNA sequence appear to encode for secreted proteins. We will raise antisera against these proteins and will confirm the presence of these proteins in sera from neurofibromatosis patients through Western blot analysis and RIA. In a more large-scale effort, we will attach immunoglobulins to solid substrates to form protein microarrays. Labeled serum protein from neurofibromatosis patients and control normal individuals will be hybridized to test for differential protein expression in the serum of these patients. Months 24-48.
NOT COMPLETED
No potential candidate serological markers that reliably distinguish between MPNST and benign tumors were identified.

Task 8 Genes that are highly expressed in MPNST and that are growth factor receptors, tyrosine kinases, or other biologically active molecules will be examined for their potential to serve as therapeutic targets. In the first analysis, primary cell cultures derived from resected MPNST specimens will be used. If not available, a search will be made through existing cell lines to find one that expresses the targeted markers, and will be used to test for biologically active growth modulation *in vitro*. Months 36-48.
NOT COMPLETED

<u>The reviewer requests an explanation of acronyms and points out that typographical errors were made.</u>
MPNST: malignant peripheral nerve sheath tumor, a sarcoma
Significance Analysis of Microarrays: method to rank order genes depending on their ability to help discern tumor categories
Plexiform: a specific type of growth pattern seen in a subset of neurofibromas
SS: synovial sarcoma
EGFR: epidermal growth factor
TRIzol: a compound used to isolate RNA

HEEBO: a new type of gene array manufactured at Stanford that uses oligonucleotides instead of cDNAs as probes
3+ reactivity: in immunohistochemistry staining experiments the grading of staining intensity was scored on a 4 grade scale where 0 represents negative staining and 1,2,3 represent increasing staining intensity.

Typographical errors:
"associateed" should be associated
"proliferaton" should be proliferation

Correction:
Our final report stated that a publication by AJ Larson et al. (Epidermal growth factor receptor (EGFR) expression and gene amplification spectrum of spindle cell soft tissue neoplasms. A fluorescence in situ hybridization (FISH) and immunohistochemical study).was in press. This has turned out to be a miscommunication between us and the senior author on that paper. We have instead incorporated the information on this marker in our manuscript that is now nearing completion and that we expect to submit in the next months.

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LEGENDS FOR FIGURES

Figure 1
Unsupervised hierarchical clustering analysis of 6 MPNSTs and 7 SS cases based on miRNA expression. Each row represents the relative levels of expression for a single miRNA and each column shows the expression levels for a single sample. The red or green color indicates relatively high or low expression, respectively, while grey indicates absent datapoints. The two main groups of the dendrogram labeled as A (MPNST) and B (SS) separated the tumors.

Figure 2
Unsupervised hierarchical clustering analysis for mRNA gene expression profiling of 3 MPNSTs and 3 SS cell line established from the fresh tumors. The MPNSTs are color coded with blue and SS with red. The expression profiles of MPNSTs and SS are compared with 25 other sarcoma samples.

Figure 3A-E, Immunohistochemistry staining for EGFR.
A) MPNST showing 3+ reactivity to EGFR, with >50% of the tumor cells having intense membrane staining.
B) Example of 2+ immunoreactivity in a plexiform NF with moderate staining in >10% of tumor cells.
C) Example of 1+ immunoreactivity in a NF with weak staining in <10% of tumor cells (magnification 200x, inset 400x).
D) Example of a synovial sarcoma with 3+ reactivity to EGFR, with >50% of the tumor cells showing diffuse membrane staining.
E) Example of a Schwannoma with no immunoreactivity to EGFR (magnification 200x)

Figure 4. Example of a MPNST with EGFR amplification. The centromeric probe (CEP7), directed against 7p11.1-q11.1, emits two green fluorescent signals per nucleus, while the EGFR probe, directed against 7p12 fluoresces orange and shows multiple copies of the EGFR gene. In this case, the EGFR/CEP7 ratio was 4.2. Example of a MPNST with polysomy 7, in this example the chromosome copy number average was 5.5.

APPENDICES

Appendix 1: Manuscript
Genome-wide transcriptome analysis identifies gene signature for malignant transformation in peripheral nerve sheath tumors (submitted)

Appendix 2: Abstract
Gene expression profiling of 24 Novel sarcoma cell lines
CH Lee, S Zhu, M van de Rijn, JA Fletcher. Stanford University, Stanford, CA; Brigham and Women's Hospital, Boston, MA Annual meeting USCAP 2007.

Appendix 3: Manuscript
Epidermal growth factor receptor (EGFR) expression and gene amplification spectrum of spindle cell soft tissue neoplasms. A fluorescence in situ hybridization (FISH) and immunohistochemical study (in press 2008).

Figure 1

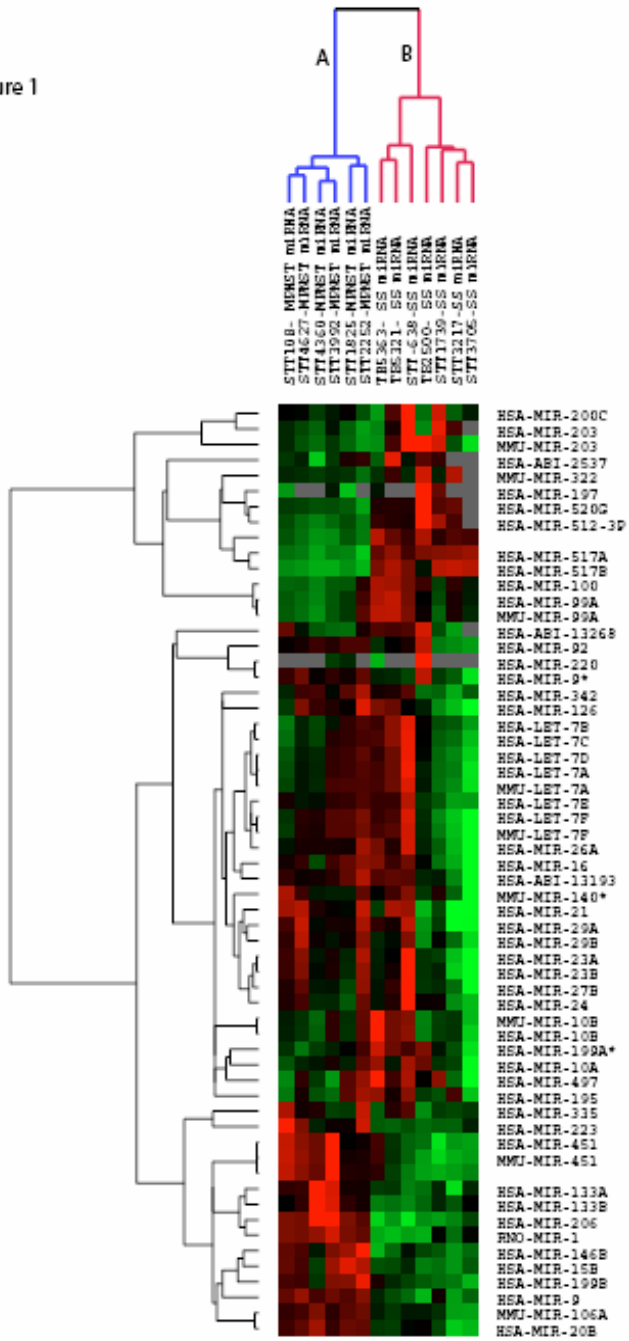
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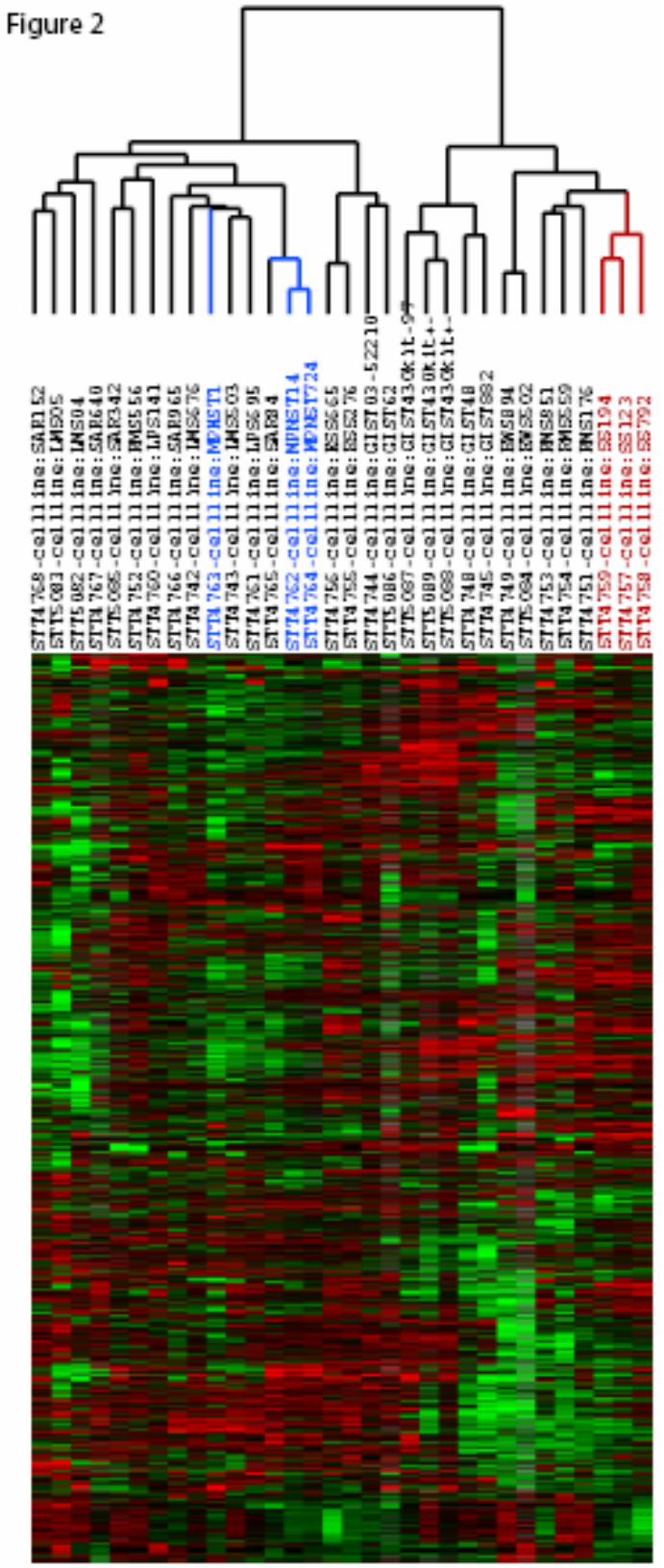
Figure 2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STT4742-cell line:LMS676
STT4763-cell line:MPNST1
STT4743-cell line:LMS503
STT4761-cell line:LPS695
STT4765-cell line:SAR84
STT4762-cell line:NPNST14
STT4764-cell line:MPNST724
STT4756-cell line:ESS665
STT4755-cell line:ESS276
STT4744-cell line:GIST03-52210
STT5086-cell line:GIST62
STT5087-cell line:GIST430k1-99
STT5089-cell line:GIST430k1+-
STT5088-cell line:GIST430k1+-
STT4748-cell line:GIST48
STT4745-cell line:GIST882
STT4749-cell line:BMS802
STT5084-cell line:BMS502
STT4753-cell line:BNS851
STT4754-cell line:BMS559
STT4751-cell line:BNS176
STT4759-cell line:SS194
STT4757-cell line:SS123
STT4758-cell line:SS792

Figure 3A-C

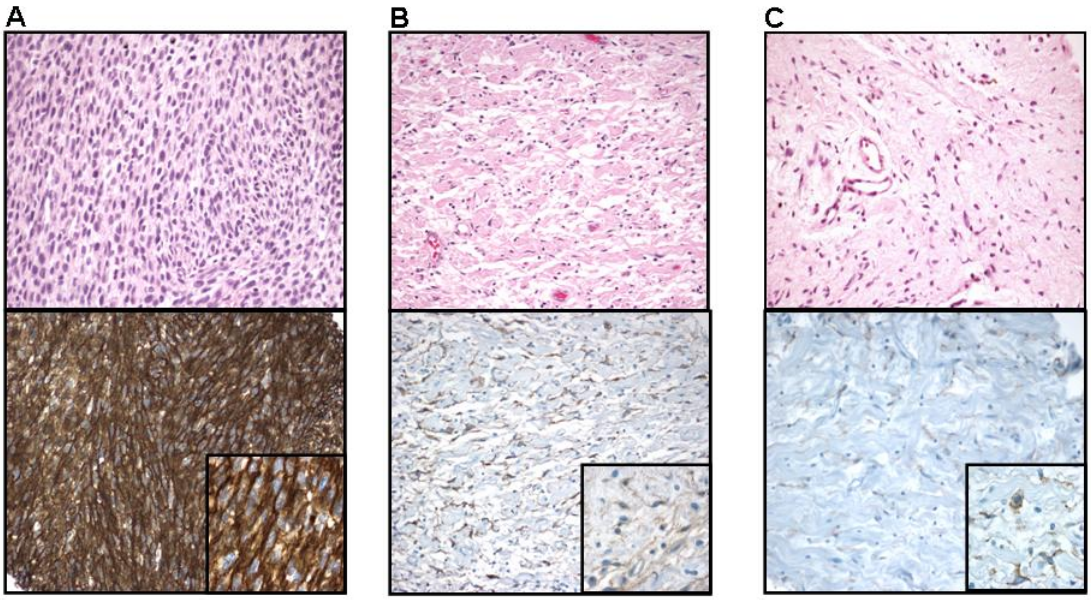

Figure 3D-E

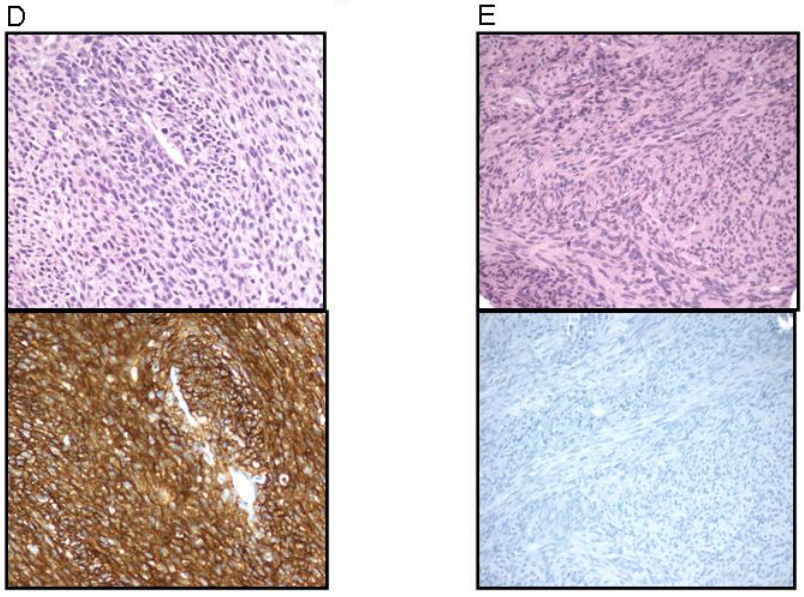

Figure 4

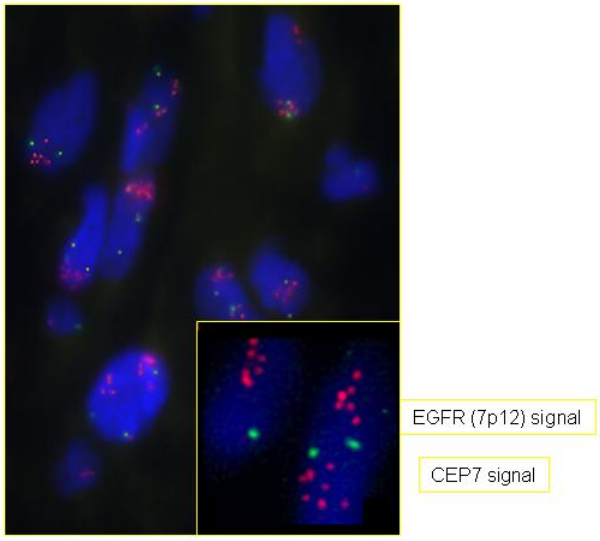

Table 1: Significance analysis of miRNA expression of MPNST

Gene ID	Score(d)	Fold Change	q-value	(%)
HSA-MIR-206	4.394659	29.62	0	
HSA-MIR-9	3.288742	5.18	0	
HSA-MIR-146B	3.000959	8.57	0	
HSA-MIR-15B	2.890168	7.84	0	
HSA-MIR-451	2.630717	7.94	0	
HSA-MIR-20B	2.62999	4.17	0	
HSA-MIR-199B	2.397392	5.70	0	
HSA-MIR-223	2.355982	7.89	0	
HSA-MIR-133A	2.350066	9.28	0	
HSA-MIR-133B	2.310541	8.00	0	
HSA-MIR-21	1.495222	2.10	0	
HSA-MIR-342	1.417306	2.38	1.94	
HSA-MIR-29A	1.351994	1.96	1.94	
HSA-MIR-29B	1.349319	2.31	1.94	
HSA-MIR-517A	-5.51924	0.09	0	
HSA-MIR-517B	-4.93106	0.06	0	
HSA-MIR-512-3P	-2.58844	0.12	0	
HSA-MIR-520G	-2.31417	0.17	0	
HSA-MIR-100	-1.99153	0.25	0	

Table2. Small RNA composition of the cDNA libraries prepared from 4 human soft tissue tumors

	SS1		SS2		NF		MPNST	
Type	no.	%	no.	%	no.	%	no.	%
previously identified miRNAs	741	90.6	637	78.0	99	56.6	206	87.3
Class I (candidate miRNA)	2	0.2	3	0.4	0	0	0	0
Class II (candidate small RNA with non-canonical hairpin)	0	0	0	0	0	0	0	0
Class III (candidate small RNA without significant hairpin)	0	0	0	0	2	1.1	1	0.4
rRNA	17	2.1	35	4.3	44	25.1	14	5.9
tRNA	18	2.2	131	16.0	13	7.4	8	3.4
sn/sno/misc-RNA	9	1.1	5	0.6	4	2.3	1	0.4
mitochondrial	24	2.9	1	0.1	0	0	0	0
repeat	2	0.2	0	0	3	1.7	0	0
mRNA	0	0.0	0	0	0	0	2	0.8
not mapped/ unknown	5	0.6	5	0.6	10	5.7	4	1.7
total number of sequences	818	100	817	100	175	100	236	100

Table 3: Annotated miRNAs of MPNSTs and SS samples

	638 (SS)		1739 (SS)		4536 (NF)		3922 (MPNST)	
miRNA	no.	%	no.	%	no.	%	no.	%
let-7a	55	7.4	4	0.6	14	14.1	12	5.8
let-7b	295	39.8	118	18.5	27	27.3	62	30.1
let-7c	32	4.3	37	5.8	1	1.0	15	7.3
let-7d		0.0		0.0	1	1.0	4	1.9
let-7d*		0.0	1	0.2		0.0		0.0
let-7e	8	1.1		0.0		0.0	1	0.5
let-7f	16	2.2	6	0.9	6	6.1	12	5.8
let-7f*		0.0	1	0.2		0.0		0.0
let-7g	5	0.7		0.0	1	1.0	2	1.0
let-7i	6	0.8	30	4.7	2	2.0	8	3.9
miR-15a	1	0.1	1	0.2		0.0		0.0
miR-16	3	0.4		0.0		0.0		0.0
miR-19b		0.0	2	0.3		0.0		0.0
miR-21	41	5.5	16	2.5	20	20.2	43	20.9
miR-22		0.0	2	0.3		0.0		0.0
miR-23a	12	1.6		0.0		0.0		0.0
miR-23b	11	1.5	2	0.3		0.0	3	1.5
miR-24	5	0.7	4	0.6		0.0		0.0
miR-25	1	0.1		0.0		0.0		0.0
miR-26a	11	1.5	1	0.2	1	1.0		0.0
miR-26b	5	0.7		0.0		0.0		0.0
miR-27a	14	1.9	2	0.3		0.0	3	1.5
miR-27b	16	2.2	1	0.2		0.0	2	1.0
miR-29a	1	0.1	1	0.2		0.0		0.0
miR-34a	1	0.1		0.0		0.0		0.0
miR-92	6	0.8		0.0	1	1.0	1	0.5
miR-92b		0.0		0.0		0.0	1	0.5
miR-93		0.0	3	0.5		0.0		0.0
miR-99a	2	0.3	5	0.8		0.0		0.0
miR-99b	7	0.9	1	0.2		0.0	1	0.5
miR-100	6	0.8	5	0.8		0.0	1	0.5
miR-103		0.0	1	0.2		0.0		0.0
miR-106b	1	0.1		0.0		0.0		0.0
miR-124a		0.0	12	1.9		0.0		0.0
miR-125a	7	0.9		0.0	2	2.0	1	0.5
miR-125b	49	6.6	16	2.5	10	10.1	11	5.3
miR-126		0.0	2	0.3		0.0		0.0
miR-126*	2	0.3		0.0		0.0		0.0
miR-127	9	1.2	41	6.4		0.0		0.0
miR-128b		0.0		0.0	1	1.0		0.0
miR-130a	4	0.5	2	0.3		0.0	1	0.5
miR-135a		0.0	1	0.2		0.0		0.0
miR-136		0.0	1	0.2		0.0		0.0
miR-140		0.0	2	0.3		0.0		0.0
miR-143	1	0.1	1	0.2		0.0		0.0
miR-146a		0.0		0.0	2	2.0		0.0
miR-148b		0.0	1	0.2		0.0		0.0
miR-151		0.0	4	0.6		0.0	1	0.5
miR-151*	25	3.4	38	6.0	7	7.1	5	2.4
miR-154	9	1.2	3	0.5		0.0		0.0
miR-181a	1	0.1	5	0.8		0.0		0.0
miR-181b		0.0	1	0.2		0.0		0.0
miR-182		0.0	2	0.3		0.0		0.0
miR-183		0.0	1	0.2		0.0		0.0
miR-185		0.0	9	1.4		0.0		0.0
miR-191	3	0.4	3	0.5		0.0	1	0.5
miR-193a	1	0.1		0.0		0.0		0.0
miR-193b	1	0.1		0.0		0.0		0.0
miR-194		0.0	1	0.2		0.0		0.0
miR-195	1	0.1		0.0		0.0		0.0
miR-196b		0.0	6	0.9		0.0		0.0
miR-197	3	0.4		0.0		0.0		0.0
miR-199a	16	2.2	141	22.1		0.0	4	1.9

miR-199a*	11	1.5	19	3.0		0.0	1	0.5
miR-199b		0.0		0.0		0.0	1	0.5
miR-200a		0.0	1	0.2		0.0		0.0
miR-200b	2	0.3		0.0		0.0		0.0
miR-200c	14	1.9	12	1.9		0.0		0.0
miR-205	1	0.1		0.0		0.0		0.0
miR-210		0.0	2	0.3		0.0		0.0
miR-214	6	0.8	9	1.4		0.0	2	1.0
miR-214*		0.0	4	0.6		0.0		0.0
miR-221		0.0		0.0	1	1.0	1	0.5
miR-320	1	0.1	13	2.0		0.0	1	0.5
miR-323		0.0		0.0		0.0		0.0
miR-324-5p		0.0	7	1.1	1	1.0	1	0.5
miR-324-3p		0.0	2	0.3		0.0		0.0
miR-335	1	0.1		0.0		0.0		0.0
miR-338		0.0	2	0.3		0.0		0.0
miR-342	1	0.1		0.0		0.0	1	0.5
miR-368	4	0.5	1	0.2		0.0	1	0.5
miR-375		0.0	3	0.5		0.0		0.0
miR-376a	4	0.5	3	0.5		0.0		0.0
miR-409-3p		0.0	1	0.2		0.0		0.0
miR-425-5p		0.0	1	0.2		0.0		0.0
miR-425-3p		0.0	1	0.2		0.0		0.0
miR-432		0.0	7	1.1		0.0		0.0
miR-450		0.0		0.0	1	1.0		0.0
miR-485-5p		0.0	1	0.2		0.0		0.0
miR-493-3p		0.0	4	0.6		0.0		0.0
miR-495	1	0.1	1	0.2		0.0		0.0
miR-497		0.0	1	0.2		0.0	1	0.5
miR-532*		0.0		0.0		0.0	1	0.5
miR-652		0.0	5	0.8		0.0		0.0
	739	**99.7**	**637**	**100**	**99**	**100**	**206**	**100**

Appendix 1.
Manuscript submitted.

Appendix 2:

Abstract

Gene Expression Profiling of 24 Novel Sarcoma Cell Lines

CH Lee, S Zhu, M van de Rijn, JA Fletcher. Stanford University, Stanford, CA; Brigham and Women's Hospital, Boston, MA Annual meeting USCAP 2007

Background: Cell lines derived from fresh tumors have been widely used as experimental models but few sarcoma cell lines exist. Since the process of culturing may alter the phenotype of the cell, a careful molecular characterization of cell lines is useful to establish their validity. The aim of our current study is to examine the gene expression profiles of 24 novel sarcoma cell lines.
Design: Oligonucleotide arrays (HEEBO, Stanford) were used to characterize the global gene expression profiles of 24 sarcoma cell lines that included 4 rhabdomyosarcomas (RMS), 2 KIT-positive gastrointestinal stromal tumors (GIST), 1 KIT-negative GIST, 3 synovial sarcomas (SS), 3 malignant peripheral nerve sheath tumors (MPNST), 2 leiomyosarcomas (LMS), 2 liposarcomas (LPS), 2 endometrial stromal sarcomas (ESS), 1 Ewing sarcoma (EWS) and 4 undifferentiated sarcomas developed at Brigham and Women's Hospital. Gene array data from 16 fresh tumors (8 GIST and 8 SS) was included for comparison. Hierarchical clustering and significance analysis of microarray data (SAM) were used for data analysis.
Results: Unsupervised hierarchical clustering of the gene array data from the 24 sarcoma cell lines showed tumor-type specific co-clustering in 12 of the 20 cell lines of known sarcoma types in 3 of 4 RMS, 3 of 3 SS, 2 of 2 KIT-positive GIST, 2 of 3 MPNST and 2 of 2 ESS. SAM analysis of the cell lines also revealed gene expression profiles in accordance with data for fresh tumor samples. More significantly, in a separate unsupervised hierarchical clustering that combined the gene array data from 16 fresh tumors (SS and GIST) together with the 24 sarcoma lines, the 2 KIT-positive GIST cell lines clustered together with the 8 fresh frozen tissue GIST while all 3 SS clustered together with the 8 fresh frozen tissue SS.
Conclusions: The results of our gene expression analysis of the cell lines reveal that the majority of the 24 sarcoma cell lines exhibit distinct tumor-specific gene expression profiles that are in accordance with the current literature. The comparison of the cell lines and fresh frozen tumor tissues in the case of GIST and SS also indicate that defining gene expression properties of synovial sarcoma and GIST are well conserved in these cell lines, thereby making them highly representative experimental models.

Appendix 3:

manuscript

EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR) EXPRESSION AND GENE AMPLIFICATION IN A SPECTRUM OF SPINDLE CELL SOFT TISSUE NEOPLASMS: A FLUORESCENCE IN SITU HYBRIDIZATION (FISH) AND IMMUNOHISTOCHEMICAL STUDY

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Running Title: EGFR in Spindle Cell Soft Tissue Neoplasms

Abstract

Epidermal growth factor receptor (EGFR) is a transmembrane glycoprotein with tyrosine kinase activity that functions in cell proliferation and survival. EGFR expression and gene amplification has been studied extensively in carcinomas and monoclonal antibodies directed against EGFR have been approved for the treatment of certain types of carcinoma. However, EGFR data in soft tissue neoplasms is limited. Using a variety of benign and malignant spindle cell neoplasms, we assessed EGFR status by fluorescence in-situ hybridization (FISH) using a dual color assay which simultaneously identifies EGFR gene copy number and the centromere of chromosome 7 (EGFR/CEP7, ratio >2 consistent with EGFR amplification; Abbott/Vysis, Abbott Park, IL) and correlated the results with EGFR expression as assessed by immunohistochemistry (IHC) using an EGFR monoclonal antibody (Ventana, Tuscon, AZ). A tissue microarray was constructed from duplicate 0.5 mm cores from formalin-fixed and paraffin-embedded tissues which included 14 synovial sarcomas (SS), 9 dermatofibrosarcoma protruberans with fibrosarcomatous change (DFSP), 5 clear cell sarcomas (CCS), 9 desmoplastic melanomas (DM), 62 malignant peripheral nerve sheath tumors (MPNST; 40 which were associated with neurfibromatosis-1 and 22 sporadic cases), 22 plexiform (PNF), 9 diffuse (DN) and 8 localized neurofibromas (LN), 21 schwannomas (SW), and 3 perineuromas (PN). Immunoreactivity was scored as negative (no staining), 1+, 2+ or 3+. Neoplasms in which the majority of samples showed 2-3+ expression by IHC included MPNST (83% of NF1-associated and 77% of sporadic), 73% of PNF, 100% DN, 56% of DFSP, 100% of PN, and 93% of SS. Of all neoplasms examined, FISH identified only 3 cases which were EGFR amplified, all of which were MPNST (2 NF1-associated, 1 sporadic; EGFR/CEP7 ratio range: 2.2-4.2) and 3 additional MPNST cases which were polysomic for chromosome 7 (3 NF1-associated, CEP7 copy number range: 3.0-5.5). All three of the CEP 7 polysomic cases showed 2-3+ immunoreactivity, while the 3 FISH amplified cases demonstrated 3+ IHC reactivity. The disparity between EGFR protein overexpression and the paucity of EGFR gene amplification may be secondary to some post-transcriptional modification, however further investigation is needed. The finding of EGFR overexpression, especially in malignant neoplasms, deserves further study as EGFR antagonists may be of benefit to patients with soft tissue tumors that express and are dependent on EGFR.

Introduction

Epidermal growth factor receptor (EGFR), located at 7p12, is a member of a protein tyrosine kinase family and functions as a cell cycle regulatory protein (Kondo and Shimizu, 1983). The receptor is activated by the binding of epidermal growth factor (EGF) and transforming growth factor-α (TGF- α), which leads to receptor autophosphorylation resulting in cell proliferaton and cell survival through inhibition of apoptosis and promotion of angiogenesis (Arteaga, 2003; Arteaga *et al.*, 1988; Hernandez-Sotomayor *et al.*, 1993). EGFR overexpression has been identified in several subsets of malignant neoplasms. For example, 40-80% of non-small cell lung carcinomas have been shown to overexpress EGFR (Rusch *et al.*, 1997).

The multiple cellular signaling interactions of EGFR biology allows for potential EGFR inhibitors to counteract cellular proliferation, angiogenesis, invasion and metastasis. Different pharmaceutical approaches have been used to target either the extracellular ligand-binding domain of the EGFR or the intracellular tyrosine kinase region (Ghosh *et al.,* 2001). The monoclonal antibody cetuximab, which inhibits ligand binding to EGFR, is currently approved for the treatment of colorectal carcinoma, and is in Phase III trials for potential treatment of other subsets of neoplasms, namely carcinomas. Erlotinib is another monoclonal EGFR inhibitor that is approved to treat patients with advanced non-small cell lung cancer, as well as first line treatment for pancreatic cancer. Recent studies have shown that responsiveness to erlotinib therapy may be linked to chromosome 7 polysomy and/or EGFR gene amplification.

There have been few studies evaluating EGFR expression and gene amplification status in sarcomas. However, EGFR immunoexpression levels have been correlated with histologic grade and poor prognosis in adult patients with soft tissue sarcomas (Sato *et al.,* 2005). It is currently unknown if down-regulation of EGFR would benefit patients with either malignant, spindle-cell soft tissue neoplasms or those patients with benign tumors which arise in locations that preclude complete resection.

Material and Methods

Archival formalin-fixed, paraffin-embedded tissues were obtained from the pathology archives of the collaborative investigators and the correlative hematoxylin and eosin stained slides were reviewed by at least three pathologists who specialize in soft tissue pathology. A tissue microarray (TMA) was constructed with duplicate 0.6 mm cores from the archival material and included MPNST from patients with neurofibromatosis (NF1-related MPNST; n=40), MPNST from patients with no history of neurofibromatosis (sporadic MPNST; n=22), monophasic fibrous synovial sarcoma (SS; n=14), clear cell sarcoma (CCS; n=5), desmoplastic melanoma (DM; n=9), typical schwannoma or cellular schwannoma (SW; n=21), localized neurofibroma (LN; n=8), plexiform neurofibroma (PNF; n=22), diffuse neurofibroma (DN; n=9), perineuroma (PN; n=3), and dermatofibrosarcoma protuberans with fibrosarcomatous change (DFSP/FS ; n=9).

Individual four micron sections of the array were deparaffinized and stained using the Benchmark Automated Slide Stainer (Ventana Medical Systems, Tucson, AZ) and the protocol established by the manufacturer for CONFIRM^{TM} Anti-Epidermal Growth Factor Receptor (EGFR) (monoclonal-clone 111.6; pre-diluted; Ventana Medical Systems, Tucson, AZ). The procedure includes incubation with Protease 2 for 4 minutes and with primary antiserum for 28 minutes. Antibody was detected using the avidin-biotin complex method, with diaminobenzidine used as the chromagen. The developed slides were counterstained with hematoxylin. Immunoreactivity was scored as negative (no staining), 1+ (weak membranous and/or cytoplasmic staining in <10% of tumor cells), 2+ (weak or partial staining in >10% or strong staining in <50% of tumor cells), or 3+ (strong staining in >50% of tumor cells).

Interphase dual-color fluorescence in situ hybridization (FISH) was performed using directly fluorescent-labeled DNA probes for the EGFR gene (7p12) with a pericentromeric control of chromosome 7 (Abbott/Vysis, Des Plaines, IL). Slides were

baked overnight and deparaffinized-dehydrated through xylene and alcohols. DAKO target retrieval was applied at 95^0 C for 40 minutes. Slides were allowed to cool to room temperature and rinsed in Milli Q water. Next, tissue was incubated for 5 minutes at room temperature with 150 uL of 1:5000 Proteinase K solution, rinsed, dehydrated with alcohols, and dried. Ten uL of probe mixture were applied and the slide was then coverslipped, codenatured at 73 C for 5 minutes, and incubated overnight. The following day the slides were subjected to stringent wash to remove any unbound probe using 2 X SSC and 0.4 X SSC/0.3% NP-40, allowed to air dry completely, and counterstained with DAPI (4', 6-diamidino-2-phenylindole). The signals were visualized on an Axioscop photomicroscope (Zeiss, Oberkochen, Germany) equipped with a triple bandpass filter. Images were captured through a photometric digital cooled CCD camera using a SmartCapture system (Vysis).

In scoring the FISH, 40 tumor nuclei were scored per core, with enumeration of both the centromeric probe, CEP7 (7p11.1-q11.1) which fluoresces green and the EGFR probe (7p12) which fluoresces orange. To avoid nuclear truncation which can occur in tissue sections, only cells containing a minimum of two centromeric signals were included. An EGFR/CEP7 ratio was calculated for each core, with a ratio of <2 equivalent to no EGFR amplification and a ratio of ≥ 2 equating with EGFR amplification.

Results

Examples of EGFR immunohistochemical staining intensities are shown in Figure 1 and the staining intensities for each entity are summarized in Table 1. Of the MPNST, 76% had 2-3+ expression by IHC, with similar results seen in both NF1-associated and sporadic MPNST cases. Of the benign nerve sheath tumors, 100% of DN, 100% of PN, and 73% of PNF demonstrated 2-3+ immunoreactivity, while only 38% of LN and 5% of SW had similar reactivity. The non-nerve sheath tumors that most often showed 2-3+ EGFR immunoreactivity included SS and DFSP (93% and 56%, respectively), while CCS and DM had no EGFR staining in greater than 50% of cases.

EGFR amplification by FISH was identified in only 3 of the neoplasms examined (Figure 2), all of which were malignant peripheral nerve sheath tumors (4.6% of MPNST EGFR amplified; 2 NF1-associated, 1 sporadic; EGFR/CEP7 ratio range: 2.2-4.2). Polysomy of chromosome 7 was identified in 3 additional cases, all of which were NF1-associated malignant peripheral nerve sheath tumors (chromosome 7 copy number range: 3.0-5.5). The FISH-amplified cases demonstrated 3+ IHC reactivity and the CEP 7 polysomic cases showed 2-3+ immunoreactivity. All other neoplasms had no evidence of EGFR amplification (EGFR/CEP7 ratio range: 1.0-1.8).

Discussion

A subset of both benign and malignant spindle cell lesions included in this study showed significant protein overexpression (2-3+ immunoreactivity) of EGFR. EGFR gene amplification by FISH was only identified in rare examples of MPNST, all of which showed 3+ staining for EGFR. These findings suggest that gene amplification is not the mechanism of increased EGFR protein expression, and that transcriptional or post-transcriptional processes are likely involved. Other studies focusing on EGFR gene

amplification status in solid malignancies including lung, prostate, bladder, head and neck, and gastrointestinal tumors have all reported low incidences of gene amplification relative to receptor overexpression, implying that gene amplification is not the prevailing mechanism of EGFR overexpression (Spaulding and Spaulding, 2002). EGFR signaling may additionally be increased by a number of mechanisms in addition to high expression levels of EGFR, including receptor mutations, heterodimerization with other members of this receptor family such as HER2 (erbB2), increased expression of (autocrine/ paracrine) ligands, and alterations in molecules that control receptor signaling output (Arteaga, 2002).

An optimized immunohistochemistry assay for EGFR has been developed in colorectal, head and neck, and other tumors that are known to express EGFR, and this assay is currently being used in colorectal cancer trials of select patients for treatment (Spaulding and Spaulding, 2002). However, to date, no clear association has been established between EGFR expression levels and response to EGFR-targeted agents.

The results reported here of EGFR expression and genomic amplification status in soft tissue tumors parallel the findings in the majority of studies evaluating EGFR expression/amplification in carcinomas. Subsets of patients with malignant soft tissue neoplasms or those with benign tumors which arise in locations which preclude complete resection may be suitable candidates for trials with anti-EGFR chemotherapeutics.

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